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Alexandria, VA 22313-1450

Attachment B

Exhibit 5

Fischer et al. (2000), "Neutrophil Elastase Induces *MUC5AC* Messenger RNA Expression by an Oxidant-Dependent Mechanism." CHEST, 117(5):317S-319S.

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Neutrophil Elastase Induces *MUC5AC* Messenger RNA Expression by an Oxidant-Dependent Mechanism*

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(CHEST 2000; 117:317S–320S)

Airway diseases such as cystic fibrosis, chronic bronchitis, and viral- or pollution-triggered asthma have two common pathologic features: mucus obstruction of the airways, and neutrophil-predominant airway inflammation. Neutrophils release high concentrations of elastase (neutrophil elastase [NE]), a serine protease, into the airways; exposure to elastase results in secretory metaplasia and increased production/secretion of mucin glycoproteins. We have pre-

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viously shown that NE increases gene expression of a respiratory mucin, *MUC5AC*, in both A549, a lung adenocarcinoma cell line, and cultured normal human bronchial epithelial cells. In this study, we explored the intracellular signaling mechanisms required for NE-regulated *MUC5AC* gene expression. A549 cells were treated with dimethylthiourea (DMTU; 4 mM and 40 mM), a scavenger of hydroxyl radical, peroxynitrite, and other hydroxylated products, prior to and during NE stimulation. DMTU inhibited NE-induced *MUC5AC* expression. Furthermore, using dichlorodihydrofluorescein, an intracellular redox indicator, we showed that in both A549 cells and cultured normal human bronchial epithelial cells, NE treatment induced oxidative stress. These results support the role of reactive oxygen species mediating NE-induced *MUC5AC* gene expression.

Key words: airway epithelium; elastase; *MUC5AC*; oxidative stress

Abbreviations: DCHF = dichlorodihydrofluorescein diacetate; DMTU = dimethylthiourea; NE = neutrophil elastase; NHBE = normal human bronchial epithelial cells; ROS = reactive oxygen species

Patients with chronic airway diseases such as cystic fibrosis, chronic bronchitis, and asthma are plagued by recurrent airway inflammation and obstruction leading to respiratory impairment/failure. In these airway diseases, the predominance of a neutrophilic inflammation suggests an important role for neutrophils and their associated products in the pathogenesis of the airway injury. Neutrophils release a variety of inflammatory mediators, including reactive oxygen species (ROS) and neutrophil elastase (NE). NE is a serine protease that has been shown to impair ciliary function, stimulate mucus production and hypersecretion, and induce mucus cell hypertrophy and hyperplasia.[1] However, the mechanism(s) of NE-induced mucus production are not known. We suggest that NE increases mucus production by inducing the expression of mucin genes.

Mucin glycoproteins are the major macromolecular components of mucus. Mucins are large, heavily glycosylated molecules; to date, at least nine different mucin genes have been identified. Of the mucin genes expressed in the airway tissues, *MUC5AC* appears to be one of the major respiratory mucins.[2] We have recently reported that NE increases respiratory epithelial expression of *MUC5AC* messenger RNA by increasing *MUC5AC* messenger RNA stability.[3] In this study, we explored the intracellular signaling mechanisms required for regulation of *MUC5AC* expression by NE. We report that NE induces the production of ROS that mediate the regulation of *MUC5AC* gene expression.

Materials and Methods

Cell Culture

Two respiratory epithelial model systems were utilized for these studies: A549 cells (American Type Culture Collection; Manassas, VA), a lung adenocarcinoma cell line that expresses both *MUC5AC* messenger RNA and glycoprotein; and normal human bronchial epithelial cells (NHBE; Clonetics; San Diego, CA) maintained in an air-liquid interface culture system. Both cell types were grown and maintained as previously described.[3]

Cell Stimulation

All studies were performed when A549 cells are 90 to 95% confluent. Cells were changed to serum-free medium. A549 cells were pre- and co-incubated with dimethylthiourea (DMTU; Sigma; St. Louis, MO), 4 mM and 40 mM, and then treated with 100 nm (2.6 U) NE or vehicle control (Elastin Products; Owensville, MO) for 6 h. Total cellular RNA was isolated, and *MUC5AC* messenger RNA expression was evaluated by Northern analysis.

RNA Isolation and Northern Analysis

Total cellular RNA was isolated from cell cultures as previously described by the guanidinium thiocyanate-cesium chloride method.[3] Total RNA was separated by agarose-formaldehyde gel electrophoresis, transferred to nylon membrane (Nytran plus; Schleicher & Schuell; Keene, NH), cross-linked, and probed with ^{32}P-labeled complementary DNA probe for *MUC5AC* as previously described.[3]

Assessment of Cellular Oxidative Stress Utilizing Dichlorodihydrofluorescein

For the evaluation of NE-induced cellular oxidative stress, cells were loaded with dichlorodihydrofluorescein diacetate (DCHF; A549, 2.5 μM; NHBE, 10 μM; Molecular Probes; Eugene, OR). Cells were exposed to NE or vehicle control. (A549, 100 nm, 30 min.; NHBE, 500 nm, 60 min.) and then evaluated for evidence of oxidative stress by fluorescent photomicroscopy. Hydrogen peroxide (1 mM) was used as a positive control stimulus for oxidative stress.

Results

NE-Induced MUC5AC *Messenger RNA Expression Is Inhibited by Oxidant Scavengers*

NE increases *MUC5AC* messenger RNA expression by enhancing messenger RNA stability, but the signaling pathway of this mechanism is not known. We hypothesized that ROS mediate the NE-regulated increase in *MUC5AC* messenger RNA stability. To test this hypothesis, we treated cells with DMTU, a scavenger of hydroxyl radical, associated hydroxylated products, and peroxynitrite[4] prior to and during NE stimulation. DMTU had no effect on baseline *MUC5AC* messenger RNA. Importantly, DMTU (40 mM) inhibited NE-induced *MUC5AC* expression (Fig 1).

NE-Induced Oxidant Stress in Respiratory Epithelial Cells

The effect of DMTU on NE-induced *MUC5AC* expression suggests that NE may function by inducing cellular oxidative stress as part of the regulatory mechanisms important for enhancing *MUC5AC* messenger RNA levels. Therefore, to evaluate if NE triggers ROS production and cellular oxidative stress in A549 cells and NHBE, we

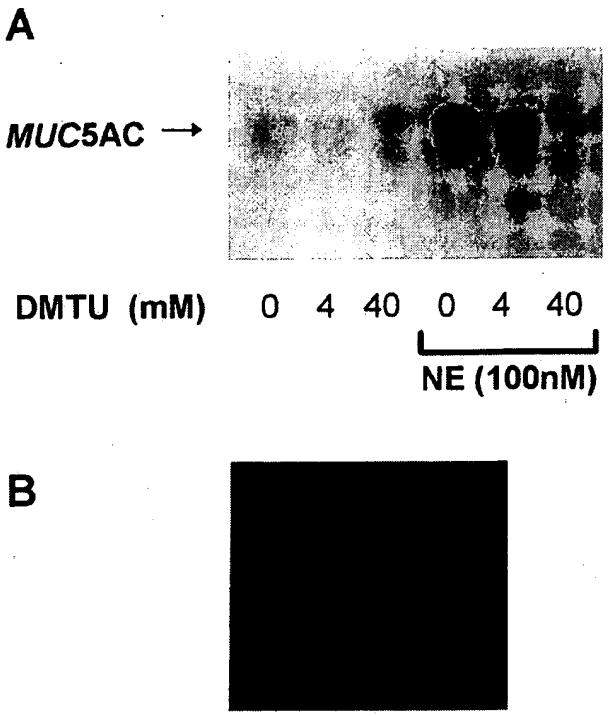

FIGURE 1. NE-induced *MUC5AC* expression: inhibition by ROS scavenger DMTU. A549 cells were treated with DMTU (0, 4, or 40 mM) 30 min prior to and during NE treatment (0 nm or 100 nm, 6 h). *MUC5AC* messenger RNA expression was evaluated by Northern analysis (*top, A*). Ethidium stain of agarose gel (*bottom, B*).

used a fluorescent intracellular redox indicator, DCHF. Cells were loaded with DCHF. In the presence of ROS such as hydrogen peroxide, DCHF is oxidized to the fluorescent product dichlorofluorescein.[5] Fluorescent microscopy revealed that NE and exogenous hydrogen peroxide increased cellular fluorescence. There was no increase in fluorescence in cells treated with control vehicle, and there was no autofluorescence in cells not loaded with DCHF. These studies together implicate the role of oxidative stress in the regulation of NE-induced *MUC5AC* expression.

DISCUSSION

In this study, we demonstrated that NE enhancement of *MUC5AC* messenger RNA levels was dependent on the production of intracellular oxidants or an alteration in the redox state of the cell. DMTU inhibited NE-induced *MUC5AC* expression, suggesting a role for hydroxyl radical, hydroxylated products, or peroxynitrite in *MUC5AC* gene regulation. Recently, we have reported that NE increases *MUC5AC* messenger RNA levels by a posttranscriptional mechanism.[3] Collectively, this suggests that ROS may play a role in *MUC5AC* messenger RNA stability. Posttranscriptional regulation of gene expression by ROS is not unique to *MUC5AC*. ROS have been reported to mediate increased messenger RNA stability of vascular endothelial growth factor,[6] catalase,[7] and the transferrin receptor.[8] It is possible that NE treatment of respiratory epithelial cells affects similar redox or ROS-sensitive protein interactions with *MUC5AC* messenger RNA stability sequences.

Using a fluoroscopic assay to detect intracellular oxidant stress, we demonstrate that in both A549 cells and NHBE, NE treatment induced oxidant stress. The sources of NE-triggered ROS production and oxidant stress are not known in epithelial cells, but there are several potential mechanisms including: generation by oxidases, lipoxygenases, P450 activity or "leakage" of mitochondrial oxidants.[9] NE has also been reported to stimulate intracellular oxidant stress in endothelial cells by catalyzing the conversion of xanthine dehydrogenase to xanthine oxidase, a superoxide generator.[10] There may be a similar mechanism activated by NE in epithelial cells. Our report suggests that NE should be included in a growing list of inflammatory mediators—including platelet-activating factor,[11] tumor necrosis factor-α, and lipopolysaccharide[12]—that stimulate increased intracellular oxidant stress.

In summary, NE treatment resulted in increased *MUC5AC* messenger RNA expression mediated by ROS. In addition, NE induced cellular oxidative stress. These results suggest that ROS may play an important role in NE regulation of *MUC5AC* messenger RNA stability.

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